Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Beck LH Jr, Bonegio RGB, Lambeau G, et al. M-type phospholipase A_2 receptor as target antigen in idiopathic membranous nephropathy. N Engl J Med 2009;361:11-21.

SUPPLEMENTARY APPENDIX

DETAILED METHODS

HUMAN KIDNEY TISSUE

Deceased donor human kidneys that were unsuitable for transplantation were obtained from the New England Organ Bank. Upon arrival (either on ice or on a perfusion pump), kidneys were stripped of their capsule and cortex was removed and minced. These cortical sections were used immediately or snap frozen and stored in liquid nitrogen for later processing. Glomeruli were collected using a series of graded sieves (Fisher Scientific) [1]. Fresh or thawed cortical sections were pressed through the top sieve with a flat plastic pestle, and copious amounts of cold phosphate buffered saline, pH 7.4 (Boston BioProducts) were used to wash the glomeruli free of the majority of the tubular and interstitial elements. Glomeruli were washed three times with cold PBS, and the resultant preparation consisted of approximately 80-85% glomeruli. The glomerular pellet was initially resuspended in an equal volume of 100 mM Tris, pH 8, 1 mM $MgCl_2$ and freeze-thawed at -80° C. An equal volume (twice the glomerular pellet volume) of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS; Boston BioProducts) was added with 1x Protease Inhibitor Cocktail Set I (Calbiochem / EMD Chemicals, Inc.) and the glomeruli were manually Dounce homogenized while on ice. Glomerular proteins were extracted on ice for 40 min, with intermittent vortexing. RIPA-insoluble debris was removed by a 10 min centrifugation at 14,000 rpm at 4° C. Due to the presence of contaminating human IgG in this preparation, the human glomerular extract was incubated for 4 h (or overnight) at 4° C with

Immobilized Protein G Plus (Thermo Fisher) and the beads discarded. Human glomerular extract was tested by WB with anti-human IgG to ensure that all detectable IgG had been removed. A cell membrane preparation obtained by ultracentrifugation of homogenized glomerular lysates yielded identical results (not shown). Therefore, we performed all subsequent experiments with the detergent extracts of homogenized whole glomeruli.

Although not used for the figures presented in this paper, a fraction of primarily human renal tubular and interstitial cells was also recovered from the sieving process. The large volume of PBS used to wash the tubules through the final sieve was collected and pelleted using 50 ml conical tubes. The tissue pellet was washed three times with PBS and extracted as above with RIPA buffer containing protease inhibitors. We found that this tubular extract contains relatively little PLA2R (perhaps due to glomerular contamination) and large amounts of neutral endopeptidase (NEP), known to be expressed by proximal tubule brush border, when compared to the glomerular fraction.

CELL-EXPRESSED RECOMBINANT PLA2R

A human embryonic kidney (HEK) 293T cell line was used for transient transfection in order to express recombinant human M-type PLA2R. A plasmid vector containing either the full-length wild-type human PLA2R or soluble human PLA2R under the control of the CMV promoter was transiently transfected into HEK293T cells using the FuGene6 transfection reagent (Roche Diagnostics). At 48 h, cells were washed twice with PBS and lysed with RIPA buffer (350 µl per subconfluent P100 dish) plus 1x protease inhibitors as above. Cells were sheared by trituration through a 25g needle, and extracted on ice for 40

min. Insoluble cell debris was centrifuged and discarded. As a transfection control, pRL-CMV (Promega) expressing *Renilla* luciferase was used instead of the PLA2R plasmids.

IMMORTALIZED HUMAN PODOCYTES

Human podocytes immortalized via transfection of a temperature-sensitive SV40 T-antigen were obtained from R. Verma (U. Michigan Medical School) with permission of M. Saleem (University of Bristol, UK), who established the cell line [2]. They were maintained in RPMI medium (Gibco) with the addition of L-glutamine, penicillin, streptomycin, and an insulin-transferrin-selenium mixture (Gibco) at the growth-permissive temperature of 33°C. Cultured podocytes were differentiated by 14 days or more of culture at the growth-restrictive temperature of 37°C.

DEGLYCOSYLATION WITH PEPTIDE N-GYCOSIDASE F

N-linked carbohydrates were removed with peptide N-glycosidase F (PNGase F; New England Biolabs) in the absence of reducing agent. Incubation was for 2 h at 37° C. Both human glomerular extract and transfected HEK293T cell extract were treated identically.

WESTERN BLOT PROTOCOL

Samples were prepared for WB by adding one volume 4x non-reducing sample buffer (250 mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.03% Pyronin Y; Boston BioProducts) to three volumes of sample and boiling for 5 min. For certain experiments, reducing sample buffer was substituted for the non-reducing buffer, which yielded final beta-mercaptoethanol concentration of 2%. Samples were loaded into precast 4-15%

Tris-HCl Ready Gels (BioRad Laboratories) and electrophoresed in the presence of Tris-glycine-SDS running buffer. Proteins were transferred to Immobilon nitrocellulose membranes (GE Water & Process Technologies) in the presence of Tris-glycine transfer buffer containing 15% methanol, at 0.85 A. Membranes were incubated for 1 h at room temperature or overnight at 4° C in a blocking solution containing 1% bovine casein in Tris-buffered saline/0.2% Tween-20 (TBST), which was used for all primary and secondary antibody dilutions.

All incubations with human serum were performed for 2 h at room temperature or overnight at 4° C, at a dilution of 1:100 unless otherwise indicated. The several reactive sera that have been tested at multiple dilutions have yielded positive results at dilutions up to 1:2000. Horseradish peroxidase (HRP)-conjugated donkey anti-human IgG secondary antibody (Jackson ImmunoResearch Laboratories) was used at 1:40,000. When it became apparent that there was a low titer of PLA2R-reactive antibody in several of the samples that initially tested negative at 1:100, we retested all negative samples at a dilution of 1:25. To avoid a high background, after our initial overnight incubation with human serum at 1:25, we secondarily blotted with sheep anti-human IgG4 (The Binding Site) diluted to 1:3000 and detected with HRP-conjugated anti-sheep IgG. We found three additional PLA2R-reactive sera in the idiopathic MN cohort using this more sensitive method, but none in the other 3 cohorts.

The PLA2R antibody used for these experiments is a polyclonal guinea pig antibody raised against the full-length purified rabbit PLA2 receptor [3] diluted to 1:400. It recognizes the human protein under both reducing and non-reducing gel electrophoresis conditions. Its specificity has been characterized in several ways. Specificity for PLA2R

was established by inhibition of reactivity by a protein fragment unique to the CTLD4-6 of rabbit M-type PLA2R in immunofluorescence studies. The human and rabbit M-type PLA2R share 86% identity and 92% similarity in this region. Conversely, the human PLA2R shares only 33, 32, and 27% identity and 53, 47, and 44% similarity with human Endo180, Dec205, and the mannose receptor, respectively. Donkey anti-guinea pig peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) was used at 1:20,000. Detection of human IgG subclasses was performed in a three step procedure. The reactive MN serum was incubated as above with the nitrocellulose-bound native or recombinant human PLA2R. Sheep antibodies against the four IgG subclasses (The Binding Site) were added as the secondary antibody, at dilutions of 1:2500 (anti-IgG3), 1:3000 (anti-IgG4), and 1:4000 (anti-IgG1 and anti-IgG2), as recommended by the manufacturer. Donkey anti-sheep peroxidase-conjugated secondary antibody was used as the detecting antibody at 1:10,000 (Sigma BioSciences). Blots were incubated in chemiluminescent substrate (Boston BioProducts) for 3 min, and exposed to HyBlot CL autoradiography film (Denville Scienctific, Inc.); exposure times were typically 10-30 sec for positive bands, and up to 5 min for weak or negative bands. The film was developed using a Kodak X-OMAT 2000A processor. Exposed blots were scanned with an HP ScanJet 5370 C scanner (Hewlett Packard) and figures prepared using Photoshop Creative Suite 4 (Adobe Systems, Inc.).

PARTIAL PURIFICATION BY LECTIN AFFINITY

Three ml of human glomerular extract was diluted in phosphate-buffered saline, pH 7.4 and incubated overnight at 4° C with 1 ml of wheat germ agglutinin (WGA) agarose

beads (Vector Laboratories). The beads were washed exhaustively with TBST and lectin-bound glycoproteins were eluted using 3 ml 500 mM N-acetyl glucosamine. The eluate was dialyzed against several exchanges of TBS at 4° C prior to loading on two identical 4-15% Tris-HCl Ready Gels as above, with visible protein markers (SeeBlue Plus2, Invitrogen) flanking the lanes of interest. Proteins were electrophoresed for 3 h at 150 V. One gel was transferred and blotted with reactive MN serum as above. The other gel was washed and stored at 4° C in water under conditions to avoid contamination with human secretions or skin proteins. By measuring migration distances of the native and deglycosylated reactive bands from the origin and with the visible markers as reference, we excised the gel regions of interest from the replicate gel. The bands were stored in 20% ethanol until mass spectrometric analysis.

MASS SPECTROMETRY ANALYSIS AND DATA INTERPRETATION

We excised gel regions of interest and performed in-gel tryptic digestion as previously described [4]. We analyzed the resulting peptides with a modified version of a previously described method that couples liquid chromatography (LC) with tandem mass spectrometry (MS/MS) [5]. We used the acquired MS data to search the NCBI RefSeq Human database using the SEQUEST algorithm and analyzed the data with SequestSorcerer^{TM} (Sage-N Research). The enrichment or relative abundance of each identified protein was determined by normalizing the number of spectral counts matching to the protein by its predicted molecular weight. This value has been termed a Protein Abundance Factor (PAF) [5].

IMMUNOPRECIPITATION

Fifty µl of each reactive or non-reactive human serum was incubated with 250 µl of human glomerular extract at for 2 h at 4° C, followed by the addition of 50 µl of Immobilized Protein G Plus and further incubation for 4 h. The immunoprecipitates were collected by centrifugation and extensively washed with Tris-buffered saline, pH 7.4 to remove non-specifically bound proteins, resuspended and boiled in reducing loading buffer, and electrophoresed on polyacrylamide gels. As an additional control, we omitted the human serum to exclude non-specific binding of the antigen to the Protein G beads. Immunoprecipitates were western blotted with guinea pig anti-PLA2R as above.

IMMUNOHISTOLOGY

We cryosectioned fresh frozen blocks of normal human kidney and de-identified MN or lupus MN kidney biopsies in Tissue-Tek OCT Compound (Ted Pella, Inc.) at 2 or 4 microns, fixed and permeabilized them with 1:1 methanol:acetone, and blocked them with 10% bovine serum albumin in Tris-buffered saline, pH 7.4. To detect PLA2R, we used guinea-pig anti-rabbit PLA2R at 1:150-1:200 and Cy3-conjugated donkey anti-guinea pig IgG (Jackson ImmunoResearch Laboratories) at 1:500 or AlexaFluor 488-conjugated donkey anti-guinea pig IgG at 1:500. To demonstrate specificity of the staining, we preabsorbed the polyclonal antibody with a recombinant fragment of rabbit PLA2R containing the 4^{th} to 6^{th} C-type lectin-like domains (CTLD) at a concentration of 25 µg/ml. This fragment was produced in baculovirus and purified on an OS2 sPLA2 affinity column [6]. This process significantly depleted the immunofluorescence signal to PLA2R. Additional controls for the GP anti-PLA2R included normal (pre-immune) GP

serum and an irrelevant GP anti-insulin antibody (Invitrogen). The glomerular basement membrane (GBM) proteoglycan agrin was detected with a polyclonal rabbit anti-human agrin antibody at 1:25 (Santa Cruz Biotechnology) followed by AlexaFluor 488-conjugated donkey anti-rabbit IgG at 1:500 (Invitrogen). We detected IgG4 in the MN immune deposits with sheep anti-IgG4 at 1:500 (The Binding Site) followed by FITC-conjugated (at 1:250) or Cy3-conjugated (at 1:500) rabbit anti-sheep IgG (both from Chemicon International). Single and dual stained immunofluorescence images were obtained on a Nikon TE-2000 inverted microscope (Nikon Instruments, Inc., Melville, NY) and appropriate filters linked to a Peltier cooled CCD camera (CoolSNAP HQ; Photometrics, Roper Scientific). Images were captured using NIS-Elements software (Nikon). Confocal microscopic analysis was performed with a PerkinElmer UltraVIEW LCI system using the UltraVIEW software, version 5.2. The biopsy specimen depicted in Fig. 6I and a MN biopsy control were stained with Alexa Fluor 488-conjugated anti-GP IgG to detect PLA2R and Cy3-conjugated anti-sheep IgG to detect IgG4. The image was false-colored using Adobe Photoshop to match the color scheme in Fig.6 A-H.

IMMUNOGLOBULIN ELUTION FROM BIOPSY SPECIMENS

Frozen remnant cores of de-identified human biopsy specimens (idiopathic MN, n=8; membranous lupus nephritis, n=2; IgA nephropathy, n=4) were paired (according to pathological diagnosis) to yield sufficient eluted IgG for analysis. The entire core of each specimen was sliced into 20 μm sections that were collected in a microcentrifuge tube. The sections were washed several times with TBS to remove residual OCT compound. 60 μl of 25 mM citrate buffer, pH 3.2 was initially incubated with each sample for 20 min

and collected after centrifugation. A second eluate resulting from a 20 min incubation of 40 µl 25 mM citrate buffer, pH 2.5 with the tissue sample was added to the first, and the 100 µl of eluate was neutralized with a small amount of 2 M NaOH and 2M Tris, pH 7.3. Human glomerular extract and rPLA2R were electrophoresed and transferred as described above, and small "mini-blots" containing the 125-200 kDa regions of these two lanes were cut from the larger nitrocellulose blot and blocked with 1% casein/TBS. The neutralized, eluted IgG was added to each of 7 mini-blots and incubated for 4 h in a moistened chamber. The blots were washed as above and detected with HRP-conjugated anti-human IgG at 1:40,000. The negative blots (with IgG eluted from LMN and IgAN biopsies) were reprobed with anti-PLA2R to confirm the presence of the antigen (Supplementary Figure 8). Sufficient IgG was eluted from one of the MN biopsy pairs (MN Bx3) to perform an immunoblot on a full lane of human glomerular proteins. No bands other than those detected on the mini-blots were found. We also immunoblotted HGE and rPLA2R with IgG eluted from normal human glomeruli and did not detect any bands (not shown).

POLYMERASE CHAIN REACTION (PCR) FOR PLA2R IN HUMAN PODOCYTES AND ISOLATED GLOMERULI

Total RNA was prepared from cultured immortalized human podocytes or from freshly sieved human glomeruli using an RNeasy Mini kit (Qiagen) and reverse transcribed to cDNA with oligo-dT primers using standard methodology. The forward and reverse primers for human M-type PLA2R were 5'-GAACTGCAAGCAAGCAAACA-3' and 5'-CTTGCATCTGGCATGAAGAA-3', respectively. This yields a 605 bp product present

in both the full-length and soluble PLA2R transcripts. The PCR was carried out with 35 cycles of 95° C for 30 sec, 56° C for 30 sec and 70° C for 45 sec using a GeneAmp PCR System 9700 (PE Applied Biosystems). As positive and negative controls, we used a plasmid vector containing the full-length human cDNA for PLA2R or nuclease-free water, respectively. PCR products were electrophoresed on an ethidium-containing 1% agarose gel with Tris-borate-EDTA buffer.

ASSAY FOR CIRCULATING PLA2R AND PLA2R-CONTAINING IMMUNE COMPLEXES

Since an mRNA transcript that encodes a soluble isoform of human PLA2R has been reported [7], we determined if human serum contains soluble PLA2R that could contribute to the formation of circulating immune complexes. Since we expected low amounts (if any) of sPLA2R, we first concentrated serum glycoproteins by incubating MN or control sera, diluted 1:10 in PBS, with wheat germ agglutinin (WGA) beads. As a positive control, cell-expressed recombinant soluble PLA2R was added to normal serum. The WGA beads were collected by centrifugation and washed abundantly with PBS. The beads and adsorbed glycoproteins were boiled in loading buffer containing beta-mercaptoethanol, electrophoresed, transferred to nitrocellulose, immunoblotted with anti-PLA2R and detected with HRP-conjugated anti-guinea pig IgG.

In a separate set of experiments designed to assess the ability to detect small (non-PEG-6000-precipitable; see below) PLA2R/anti-PLA2R complexes, MN or control sera diluted 1:10 in PBS were incubated with (or without) native full-length or recombinant soluble PLA2R. Protein G beads were then added to adsorb human IgG and any

antibody-bound proteins. The protein G beads were then pelleted, washed, electrophoresed, and immunoblotted for PLA2R as above.

To detect circulating immune complexes, we followed the protocol outlined by Brandslund and colleagues [8]. In brief, 200 µl of serum and 800 µl of a pH 8.3 borate buffer (100 mM boric acid, 25 mM sodium tetraborate, 75 mM sodium chloride, 0.1% sodium azide) were incubated for 90 min with 10 ml of 2.75% polyethylene glycol (PEG)-6000, and centrifuged 20 min at 1700 x *g*. The pellet was washed twice with 5 ml of 3.5% PEG-6000 and resuspended in loading buffer containing beta-mercaptoethanol. Samples were electrophoresed and immunoblotted with anti-human IgG or anti-PLA2R.

PATIENT DIAGNOSIS AND CLASSIFICATION

The diagnosis of membranous nephropathy was established by renal biopsy in all cases. Those classified as having idiopathic MN had no evidence of secondary features, which include positivity (at greater than 1:80) for anti-nuclear antibodies (ANA) or anti-double stranded DNA, hepatitis B antigenemia, or electron-dense deposits on renal biopsy in locations other than subepithelial. We did not make an attempt to rule-out occult malignancy as a potential cause of secondary MN. It should be noted that cases in which there was a possibility (but none of the features listed above) of a secondary form were conservatively classified as idiopathic MN for the purpose of this study. These included a young black female (patient MN 30 in Supplementary Table 1) with some clinical features of lupus such as serositis, but with only a high-normal titer ANA and absence of anti-double stranded DNA. Her biopsy did not show mesangial or subendothelial deposits and she has not been diagnosed with lupus according to standard rheumatologic criteria.

A Caucasian male (patient MN 32) with a long history of rheumatoid arthritis being treated with sulfasalazine (but never with any of the other anti-rheumatic drugs associated with MN) was also classified as having idiopathic MN.

The other glomerular disorders were diagnosed by biopsy and/or by clinical features. Diabetic nephropathy (1 biopsy and 3 clinical diagnoses) was determined by the presence of progressive albuminuria in the context of longstanding type 2 diabetes with suboptimal glycemic control. Primary or secondary focal and segmental glomerulosclerosis (FSGS) was largely biopsy-proven (4 patients) and suspected in another patient with obesity and obstructive sleep apnea that developed nephrotic-range proteinuria with minimal lower extremity edema. The two cases of IgA nephropathy / Henoch-Schönlein purpura were both biopsied. Two other proteinuric patients were felt to have orthostatic proteinuria (as measured by upright and supine urine collections) and hepatitis C-related nephritis (hematuria, hypocomplementemia, rheumatoid factor positivity) respectively on clinical grounds. The patients with additional autoimmune or rheumatologic conditions included systemic lupus erythematosus without significant proteinuria (n=3), dermatomyositis (n=1), mixed connective tissue disease (n=1), bullous pemphigoid (n=1), and scleroderma (n=1).

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Supplementary Table 1

Sample	Age	Race/ Ethnicity	Sex	Cr mg/dl	Alb g/dl	Urine protein	Months since biopsy	Treatment	Diagnosis
Idiopathic Membranous Nephropathy (PLA2R-reactive)									
MN1	59	Cauc	M	nl	2.1	5	NA	Cy,P,M	Idiopathic MN
MN2	47	Black	F	0.9	4.0	1.4	11	Cy,P	Idiopathic MN
MN3	49	Cauc	M	1.8	3.4	7.8	1	Cy,P	Idiopathic MN
MN4	52	Black	F	1.5	3.2	3.8	1	None	Idiopathic MN
MN5	29	Cauc	M	1.4	3.0	14	7	M,P,Cs	Idiopathic MN
MN6	40	Cauc	M	1.2	3.2	2.6	2.5	Cs,P	Idiopathic MN
MN7	53	Cauc	F	0.8	3.4	7.4	14	Cy,P,M	Idiopathic MN
MN9	44	Cauc	M	1.0	2.5	7.3	4	P	Idiopathic MN
MN10*	63	Cauc	F	0.8	4.0	4.0	48	Cy	Idiopathic MN
MN11	41	Hisp	M	0.7	3.7	3.8	96	P	Idiopathic MN
MN12	68	Cauc	M	1.4	NA	12.6	12+	Cs,P	Idiopathic MN
MN13	21	Cauc	M	0.7	2.2	5.5	1	P	Idiopathic MN
MN14	41	Cauc	M	0.6	3.6	4.9	120	Cy,P	Idiopathic MN
MN15	41	Cauc	M	2.2	4.5	0.6	108	P,Cs	Idiopathic MN
MN16	55	Cauc	M	1.0	3.8	1.6	2	None	Idiopathic MN
MN17*	88	Cauc	M	1.2	3.5	7.7	132	None	Idiopathic MN
MN18	37	Black	F	0.7	3.3	5.0	2	None	Idiopathic MN
MN19*	57	Black	M	3.1	2.8	3.9	48	None	Idiopathic MN
MN20	47	Cauc	M	0.8	3.8	2.1	60	Cy,P	Idiopathic MN
MN21*	26	Cauc	F	0.9	4.2	1.9	72	None	Idiopathic MN
MN22	63	Cauc	M	1.0	2.1	5.7	1	None	Idiopathic MN
MN23	51	Cauc	M	0.9	3.6	8.6	3	None	Idiopathic MN
MN24	NA	NA	NA	NA	NA	NA	NA	NA	Idiopathic MN
MN25	NA	NA	M	NA	NA	NA	NA	NA	Idiopathic MN
MN26	NA	NA	M	NA	NA	NA	NA	NA	Idiopathic MN
MN37	35	Hisp	M	0.7	3.5	4.1	2	None	Idiopathic MN
Idiopathic Membranous Nephropathy (PLA2R-non-reactive)									
MN8	NA	Cauc	M	NA	NA	NA	NA	NA	Idiopathic MN
MN27	47	Hisp	M	0.8	1.8	5.0	0	None	Idiopathic MN
MN28	41	Cauc	F	0.4	3.2	2.1	9.5	None	Idiopathic MN
MN29	64	Cauc	F	1.7	NA	8.0	22	M	Idiopathic MN
MN30	28	Black	F	0.7	1.8	7.0	1	Cs	Idiopathic MN
MN31	69	Arab	M	2.2	4.3	0.8	48	Cs,P	Idiopathic MN
MN32	55	Cauc	M	1.6	3.8	0.7	0	None	Idiopathic MN
MN33	51	Black	M	2.2	3.9	1.5	13	Cy,P	Idiopathic MN
MN34	26	Asian	M	1.0	NA	1.0	26	Cs,P	Idiopathic MN
MN35	NA	NA	M	NA	NA	NA	NA	NA	Idiopathic MN
MN36	NA	NA	F	NA	NA	NA	NA	NA	Idiopathic MN
Secondary Membranous Nephropathy									
HBMN1	41	Black	F	0.6	2.8	8.4	1	None	HBV-assoc. MN
HBMN2	34	Hisp	M	0.8	NA	4.0	0	None	HBV-assoc. MN

LMN1	NA	NA	F	NA	NA	NA	NA	N/A	SLE-assoc. MN
LMN2	24	Black	F	0.5	2.2	9.1	13	Cy,P	SLE-assoc. MN
LMN3	49	Black	F	0.5	3.0	2.3	8	P	SLE-assoc. MN
LMN4	38	Black	F	0.7	2.9	5.4	20	M,P,Cy	SLE-assoc. MN
LMN5	38	Black	F	0.7	3.4	0.3	3	M,P	SLE-assoc. MN
LMN6	55	Black	F	0.7	4.1	1.3	2	None	SLE-assoc. MN
Disease Controls									
DN1	60	Black	F	2.6	3.9	7.3		None	DN
DN2	60	Hisp	F	0.8	4.0	7.9	-4	None	DN
DN3	62	Black	F	7.2	3.7	2.0		None	DN
DN4	61	Cauc	M	2.5	4.3	1.2		None	DN
FGS1	35	Cauc	M	2.5	4.4	1.1	NA	None	Primary FSGS
FGS2	46	Hisp	M	0.6	NA	5.5	36	P,M,T	Primary FSGS
FGS3	33	Cauc	F	0.6	4.0	0.04	NA	P	Primary FSGS
FGS4	54	Cauc	F	0.9	3.8	3.1		None	2ary FSGS
FGS5	57	Cauc	F	0.8	4.1	0.4	54	None	2ary FSGS
MCD1	26	Asian	M	0.8	3.8	1.2	120	P,Cs,M	MCD
MCD2	66	Black	F	1.9	2.2	13.7	2	P	MCD
IgAN1	26	Cauc	M	1.0	4.3	0.6	26	None	IgAN/HSP
IgAN2	49	Cauc	M	1.0	4.1	2.0	2	P	IgAN/HSP
DC14	52	Black	M	1.5	4.2	1.7		None	HCV-assoc. MPGN
DC15	25	Cauc	F	0.7	4.3	1.1		None	Orthostatic proteinuria
DC16	78	Cauc	M	2.9	3.4	0.1		P	Bullous pemphigoid, Stage 4 CKD
DC17	45	Cauc	M	0.9	4.7	NEG		Mtx	MCTD
DC18	20	Hisp	F	0.6	4.7	NEG		P	SLE
DC19	58	Cauc	F	0.2	4.2	NEG		Mtx,P	SLE
DC20	47	Black	F	0.6	4.1	NEG		P	Dermatomyositis / SLE overlap
DC21	52	Black	M	0.9	4	1+		P,Mtx	SLE / RA overlap
DC22	54	Cauc	F	2.4	4.3	0.24		Srl, T	Scleroderma
Normal Controls									
NC1	60	Cauc	M	1.0	4.4	NEG			
NC2	37	Cauc	M	1.0	4.8	NEG			
NC3	36	Cauc	M	NA	NA	NA			
NC4	30	Cauc	F	0.6	4.4	NA			
NC5	23	Cauc	M	NA	NA	NEG			
NC6	61	Cauc	F	1.1	NA	NEG			
NC7	51	Cauc	M	0.7	NA	NEG			
NC8	53	Cauc	M	1.0	2.8	TR			
NC9	81	Hisp	F	0.5	1.5	NEG			
NC10	40	Cauc	F	0.6	3.1	NEG			
NC11	58	Cauc	F	2.2	2.5	NEG			
NC12	66	Cauc	M	0.9	NA	1+			
NC13	42	Cauc	F	1.5	3.3	1+			
NC14	18	Cauc	M	2.2	2.2	NEG			
NC15	21	Cauc	M	0.9	3.1	1+			
NC16	53	Cauc	M	1.0	3.5	NEG			

NC17	68	Cauc	M	NA	NA	NA			
NC18	38	Cauc	F	0.7	2.6	NEG			
NC19	81	Cauc	F	1.0	3.4	NEG			
NC20	70	Cauc	F	0.6	3.8	NA			
NC21	69	Cauc	M	NA	NA	NA			
NC22	58	Cauc	F	0.7	3.4	1+			
NC23	63	Cauc	F	0.8	2.6	NEG			
NC24	62	Cauc	M	NA	NA	N/A			
NC25	58	Cauc	M	1.4	4.2	NEG			
NC26	68	Cauc	F	1.2	3.5	TR			
NC27	46	Cauc	F	0.5	2.1	NEG			
NC28	65	Cauc	F	0.7	NA	NEG			
NC29	27	Cauc	F	NA	NA	NA			
NC30	28	Cauc	M	NA	NA	NA			

Supplementary Table 1 presents relevant clinical information about all patients and controls whose sera were used in experiments for this study. The table is broken into sections that correspond to the groups defined in the bar graph in Fig. 1B of the main text. Idiopathic MN is subdivided into those patients who reacted positively with PLA2R by WB, and those who were non-reactive. Secondary MN consists of two patients with hepatitis B-associated MN and six with lupus membranous nephritis. The disease controls consist of those with proteinuric glomerular diseases and those with other autoimmune diseases, with or without proteinuria. Normal controls consist of healthy volunteers, or deceased donors with preserved renal function whose kidneys were considered unsuitable for transplantation. All sera in the PLA2R-reactive idiopathic MN group were found to be reactive at a dilution of 1:100 during our initial round of testing, with the exception of those four marked with an asterisk, which were initially negative. Three of these four (MN 10, 19, and 21) were found to be reactive upon subsequent re-testing of all initially-negative samples at a titer of 1:25. Patient MN17 was initially negative when his serum was first collected; however, at that time, he was in remission from MN that had been diagnosed a decade previously. His serum was later collected when he suffered a relapse of the nephrotic syndrome and was found to be PLA2R-reactive at the time of relapse.

Designations: MN, membranous nephropathy; HBMN, hepatitis B-associated MN; LMN, lupus membranous nephropathy; DN, diabetic nephropathy; FGS, focal glomerulosclerosis; IgAN, IgA nephropathy; MCD, minimal change disease; DC, disease control; NC, normal control.

Numbers refer to the patient number in each particular group, and may be used to identify the sera used for the Figures.

Race/Ethnicity (self-reported): Cauc, Caucasian; Hisp, Hispanic; Arab, Arabic

Serum Creatinine (Cr) is presented in mg/dl.

Serum Albumin (Alb) is presented in g/dl.

Urine protein is presented as spot urine protein-to-creatinine ratio, or for DC16-20 and all normal controls, as NEG (negative), TR (trace), or 1+ as determined by urine dipstick.
Time since biopsy reflects the time in months between the initial kidney biopsy and the date of serum collection. In the case of patient DN2, the serum was collected 4 months prior to the kidney biopsy.

Treatment indicates immunosuppressive therapy at or prior to the date of serum collection. Cy, cyclophosphamide; P, prednisone or equivalent; M, mycophenylate mofetil; Cs, cyclosporine; T, tacrolimus; Mtx, methotrexate; SRL, sirolimus; None, no immunosuppressive therapy. Most proteinuric patients were also being treated with angiotensin-converting enzyme inhibitors and/or angiotensin II receptor blockers, and diuretics.

Biopsy or Clinical Diagnosis: FSGS, focal and segmental glomerulosclerosis; HCV, hepatitis C virus; HBV, hepatitis B virus; MPGN, membranoproliferative glomerulonephritis; CKD, chronic kidney disease; MCD, minimal change disease; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis.

NA, not available

Supplementary Table 2

Mass spectrometric identification of candidate antigens in membranous nephropathy

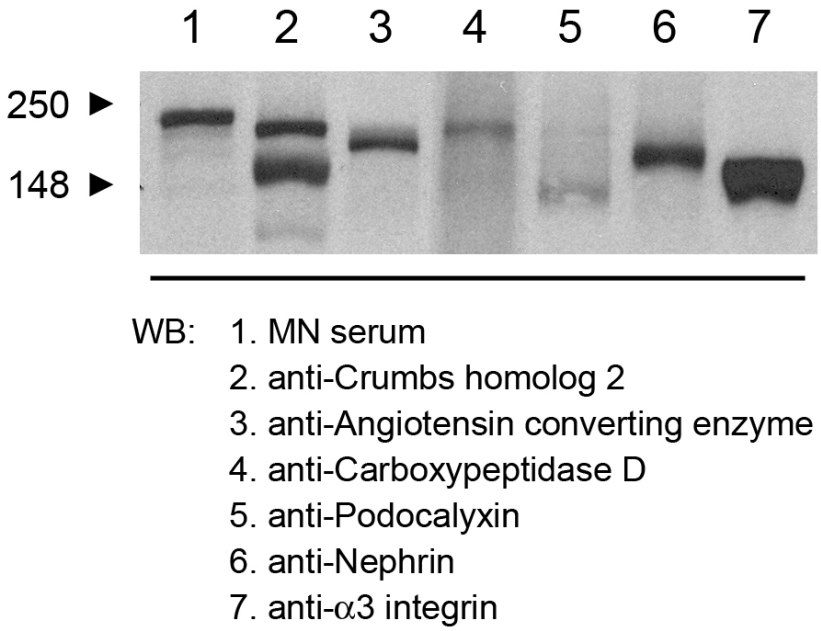
Protein	**Accession number**	**Size kDa**	**Function/ Location**	**PAF 185**	**PAF 145**
Integrin, α1 subunit	NP_852478	128	Cell adhesion molecule, ubiquitous	0.535	1.834
Integrin, α3 subunit	NP_002195	110	Cell adhesion molecule, podocyte	0.086	1.201
Endoglin isoform 2	NP_000109	65	Receptor for TGF-β family, endothelium	0.296	1.184
Aminopeptidase A	NP_001968	109	Peptidase, podocyte	0.824	0.915
Podocalyxin-like isoform 2	NP_005388	53	Constituent of glycocalyx of podocytes and endothium	0.361	0.722
Integrin, β1 isoform	NP_002202	86	Cell adhesion molecule, ubiquitous	0.296	0.566
Crumbs homolog 2	NP_775960	134	Apical polarity molecule, podocyte	0.162	0.487
Na/K ATPase α1 subunit	NP_000692	112	Subunit of transmembrane Na/K pump, ubiquitous	0.797	0.354
Membrane metallo-endopeptidase	NP_000893	85	Peptidase, podocyte		0.350
Nephrin	NP_004637	132	Slit diaphragm protein, podocyte		0.297
M-type phospholipase A2 receptor	**NP_031392**	**166**	**Receptor for PLA2, podocyte**		**0.237**
ATP synthase, H-transporting, α subunit	NP_004037	55	Subunit of mitochrondrial ATP synthase, ubiquitous	0.502	0.167
Heat shock 90 kDa protein β	NP_031381	90	Intracellular molecular chaperone, ubiquitous		0.120
Amine oxidase, Cu containing 3 precursor	NP_003725	84	Intracellular metabolic enzyme, ubiquitous		0.118
Membrane alanine aminopeptidase	NP_001141	106	Peptidase, podocyte	0.365	0.091
CD109, Gov system platelet alloantigen	NP_598000	161	GPI-linked antigen on surface of endothelium, platelets, T cells	0.062	0.062
Thrombospondin type I domain containing protein 7A; similar to KIAA0960	NP_056019	185	Not well characterized	0.212	0.042
Chondroitin sulfate proteoglycan 4	NP_01888	250	Skin, glial cells, pericytes, etc.	0.04	

Supplementary Table 2 presents a partial list of human glomerular proteins that were bound by the wheat germ agglutinin lectin column and identified by mass spectrometry. The proteins listed in the table correspond to peptides common to both the 185 kDa and 145 kDa excised gel regions. The proteins are ranked according to their PAF 145 (protein abundance factor in the 145 kDa gel region; see explanation in main Methods section). The PAF from the 185 kDa region is also listed; however, certain values are missing as they were found in an earlier mass spectrometric screen of an identical preparation, in which PAF was not calculated. Also presented are the calculated molecular weight of the mature, unmodified polypeptide sequences and the cell expression patterns. The spectra identified from PLA2R were of moderate-to-low abundance in the 145 kDa gel region, in comparison to more abundant proteins such as the integrins.

EXCLUSION OF CANDIDATE MN ANTIGENS:

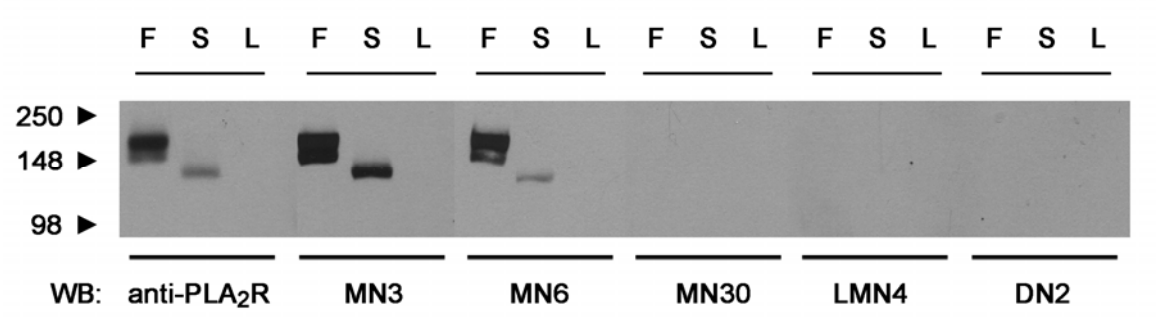
Several proteins were considered unlikely due to their size or intracellular location. Candidate proteins that were similar in predicted size to that observed for the deglycosylated MN antigen (approximately 145 kDa) and that were likely to be expressed by podocytes were evaluated further with WB and when possible by immunoprecipitation. Supplementary Figure 1 (see below) shows an immunoblot of identical lanes of human glomerular extract electrophoresed on the same gel, and blotted with PLA2R-reactive MN serum, or antibodies to crumbs homolog 3 (kindly provided by B. Margolis, U. Michigan Medical School; reacts with all mammalian crumbs homolog members), angiotensin converting enzyme (R&D Systems, Inc.), carboxypeptidase D (R. Skidgel, U. Illinois College of Medicine), podocalyxin (R&D Systems, Inc.), nephrin (kindly provided by H. Holthöfer, Dublin City University, Ireland), and α3 integrin (Chemicon International). None of these polyclonal antibodies recognize a band that is identical in size to that recognized by MN serum.

Supplementary Figure 1



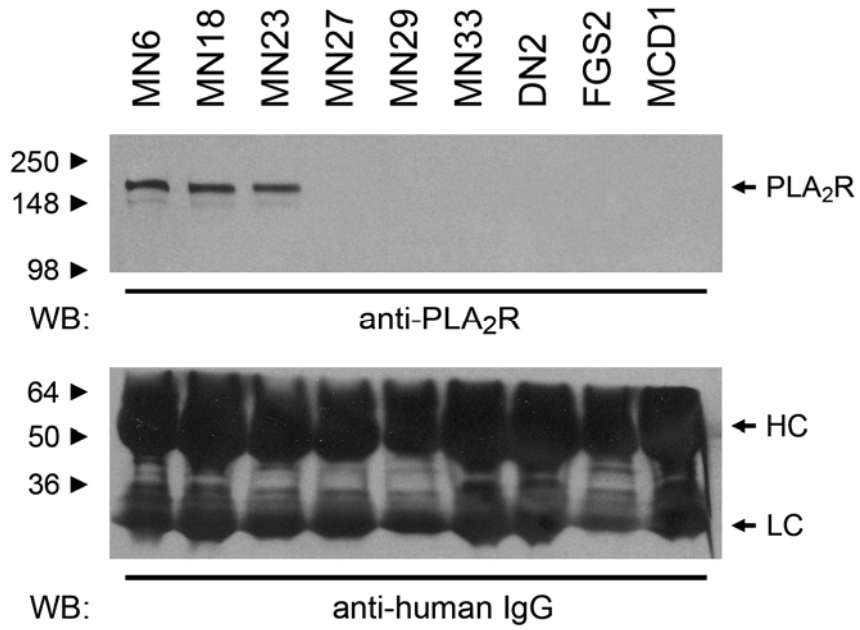
Supplementary Fig. 1: WB of human glomerular extract with reactive MN serum and antibodies to other candidate antigens as determined by mass spectrometry. Identical lanes of HGE were immunoblotted with reactive MN serum (1:100), anti-crumbs homolog (Crb) 3 cytoplasmic tail (1:1000; also detects Crb 2), anti-angiotensin converting enzyme (1:2000), anti-carboxypeptidase D (1:100), anti-podocalyxin (1:2000), anti-human nephrin (1:400), and anti-α3 integrin (1:250) and detected with the appropriate secondary antibodies. All proteins are present in protein extracts from normal human glomeruli, but none are found at the same position as the MN antigen, now identified as PLA2R.

Supplementary Figure 2A



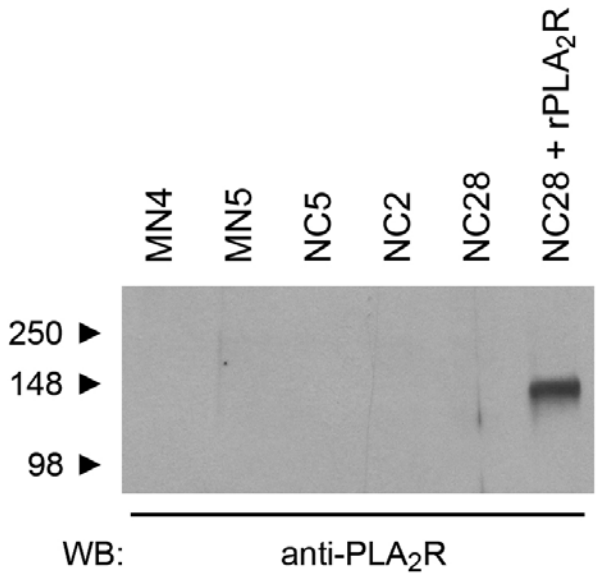
Supplementary Fig. 2A: Reactive MN sera specifically detect rPLA2R in transfected HEK293T cells. Cell extracts of HEK293T cells transfected with cDNAs encoding the full-length rPLA2R (F), the soluble isoform of PLA2R (S) or *Renilla* luciferase (L) were western blotted with sera (at 1:100) from MN patients known to reactive (MN3 and MN6) or non-reactive (MN30) with the 185 kDa glomerular antigen, or from patients with secondary MN (LMN4) or diabetic nephropathy (DN2). Human IgG4 was detected with sheep anti-IgG4 (1:3000) followed by peroxidase-conjugated anti-sheep IgG (1:20,000). The reactive MN sera specifically detect the full-length and smaller soluble PLA2R, and do not recognize any endogenous proteins from the parental 293T cell line. As a positive control, the three extracts were blotted with the monospecific GP anti-PLA2R antibody (1:500) and detected with peroxidase-conjugated anti GP IgG (1:20,000).

Supplementary Figure 2B



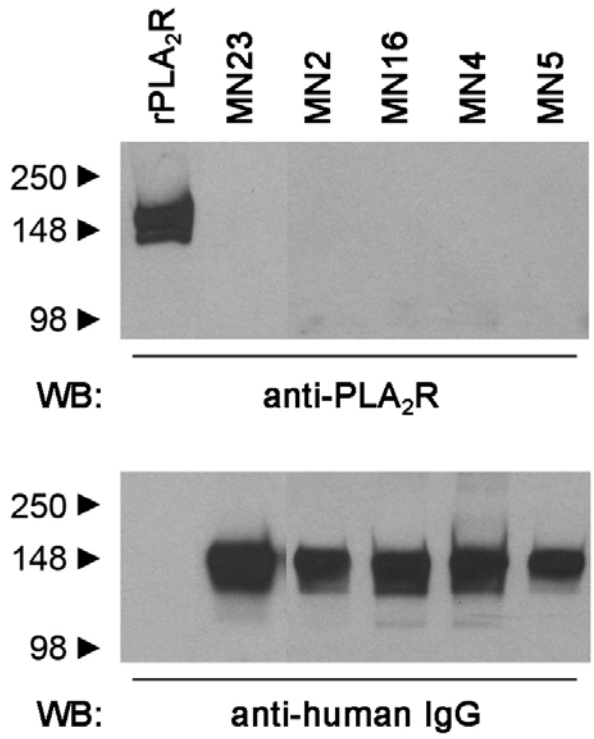
Supplementary Fig. 2B: Three reactive (MN patients 6, 18, and 23) and three non-reactive (MN patients 27, 29, and 33) sera from MN patients, as well as three control sera (diabetic nephropathy (DN) patient 2, focal and segmental glomerulosclerosis (FGS) patient 2, and minimal change disease (MCD) patient 1), were used to immunoprecipitate the target antigen from human glomerular extract. The immunoprecipitates were electrophoresed under reducing conditions and western blotted with antibodies to PLA2R (top) as well as to human IgG (bottom). As shown, all three reactive MN sera immunoprecipitate native PLA2R from human glomerular extract, whereas the non-reactive MN sera and those of controls do not. IgG heavy chain (HC) and light chain (LC) are indicated by arrows.

Supplementary Figure 3A



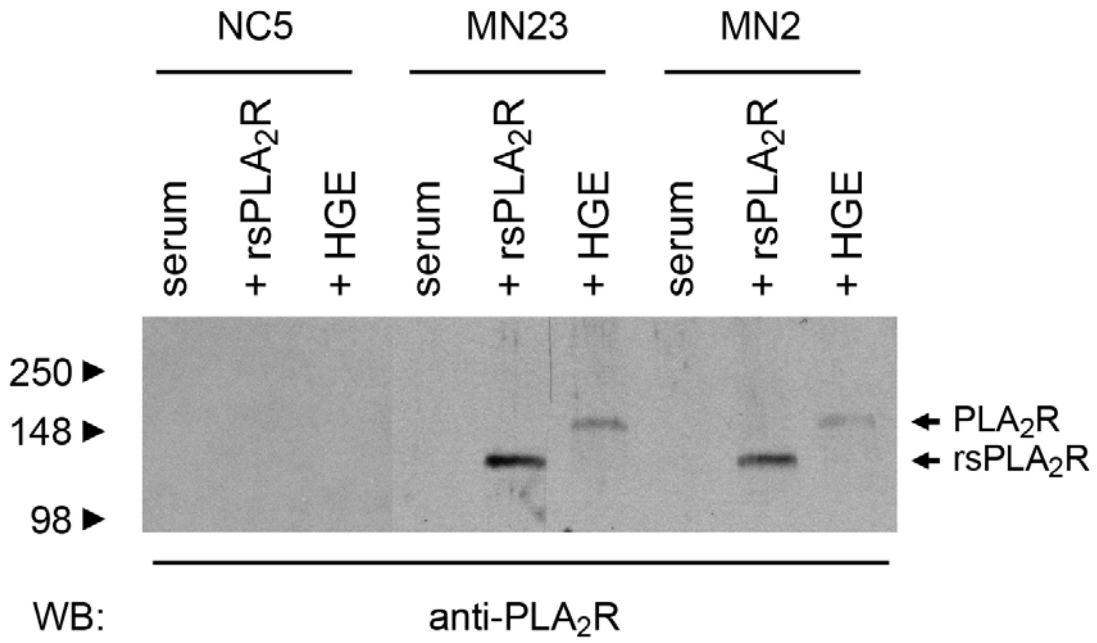
Supplementary Fig. 3A: PLA2R is not present in detectable amounts in human serum. Panel A: Serum from two MN patients (MN 4 and 5) and three normal controls (NC 5, 2, and 28) were incubated with WGA lectin beads to bind and concentrate serum glycoproteins. As a positive control to ensure that circulating PLA2R could bind WGA beads in the presence of other serum proteins, normal NC28 serum was 'spiked' with rPLA2R. After extensive washing, the WGA beads were boiled in the presence of reducing loading buffer, electrophoresed and western blotted with anti-PLA2R. PLA2R was not detected in either MN or normal control serum; it was only detected in the positive control lane.

Supplementary Figure 3B



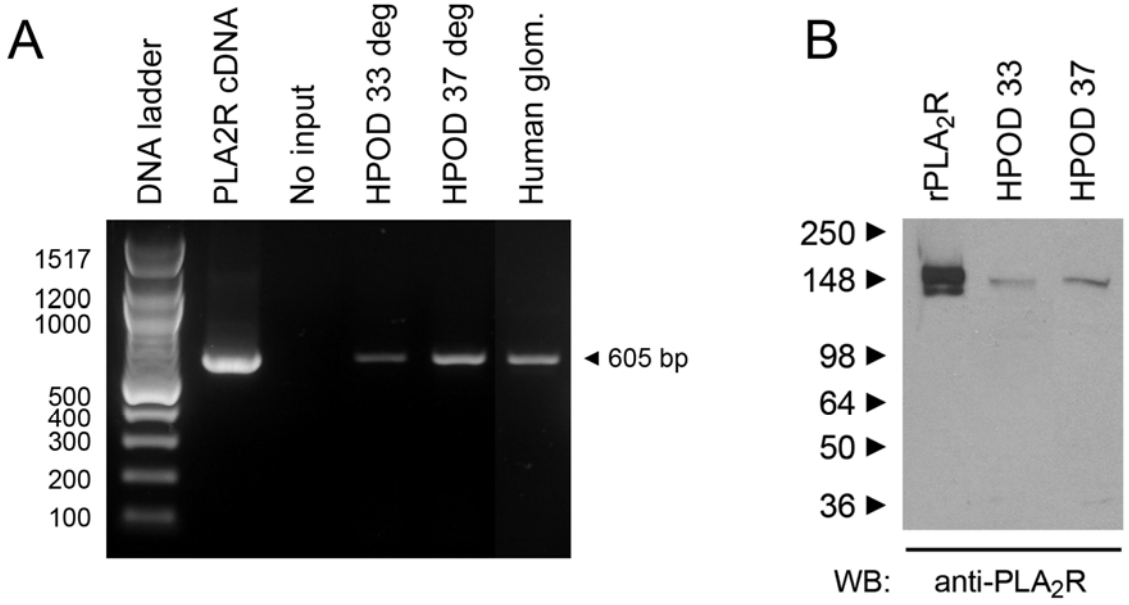
Supplementary Fig. 3B: PLA2R is not detected in PEG-6000-precipitated immune complexes (IC). PEG-6000 was used to precipitate IC from 200 µl MN sera (MN23, MN2, MN16, MN4, and MN5). The final pellets were resuspended and boiled in reducing loading buffer, electrophoresed, and successively blotted with anti-PLA2R and anti-human IgG. The leftmost lane represents a HEK293T cell lysate containing rPLA2R that was directly loaded as a positive control. Although IgG was precipitated in all cases, we did not detect PLA2R.

Supplementary Figure 3C



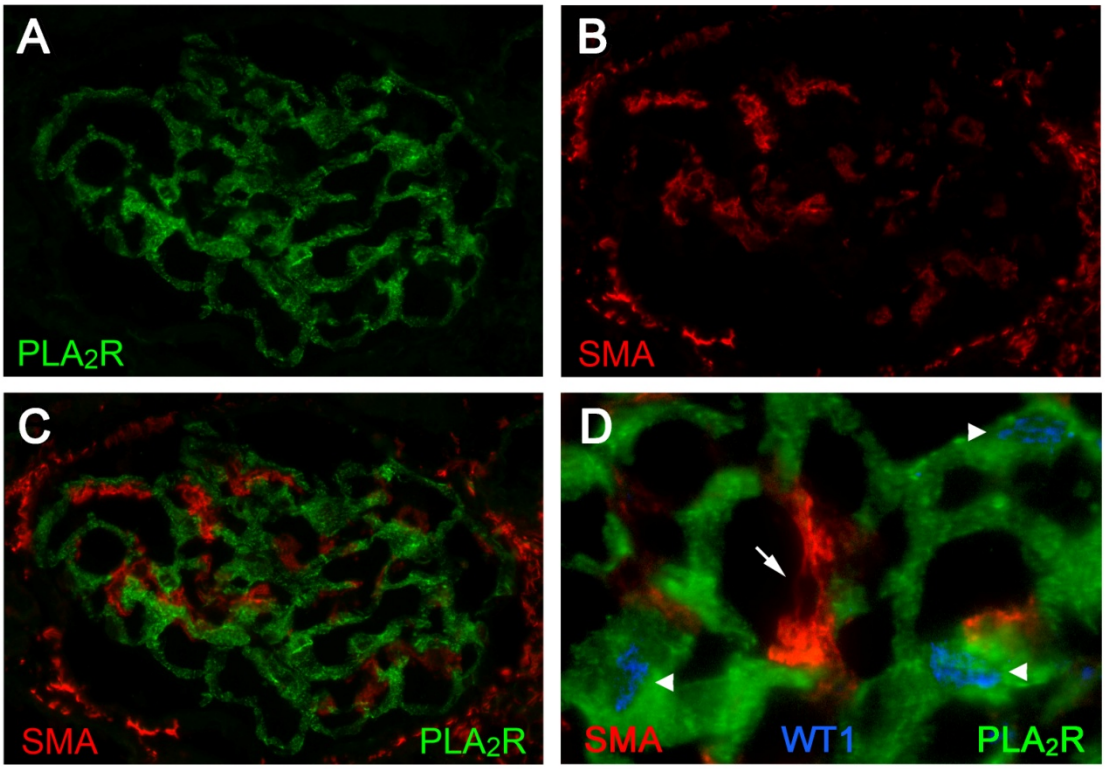
Supplementary Fig. 3C: Protein G does not immunoprecipitate PLA2R-anti-PLA2R complexes from unmodified MN serum. Because IgG4 (the major anti-PLA2R antibody) is functionally monovalent and may not be able to create large lattice immune complexes, we also attempted to immunoprecipitate small antibody-antigen complexes with protein G beads. Normal (NC5) or MN (MN23 and MN2) sera were incubated with protein G beads, either as unmodified sera, or after pre-incubation with recombinant soluble (rs)PLA2R or HGE. The immunoprecipitates were boiled in reducing loading buffer, electrophoresed, and western blotted with anti-PLA2R. PLA2R was not detected in either normal or unmodified MN sera, although it could be immunoprecipitated from MN sera if exogenous PLA2R or rsPLA2R was added. It was not precipitated from the normal control serum despite the addition of exogenous rsPLA2R because no anti-PLA2R autoantibodies were present.

Supplementary Figure 4



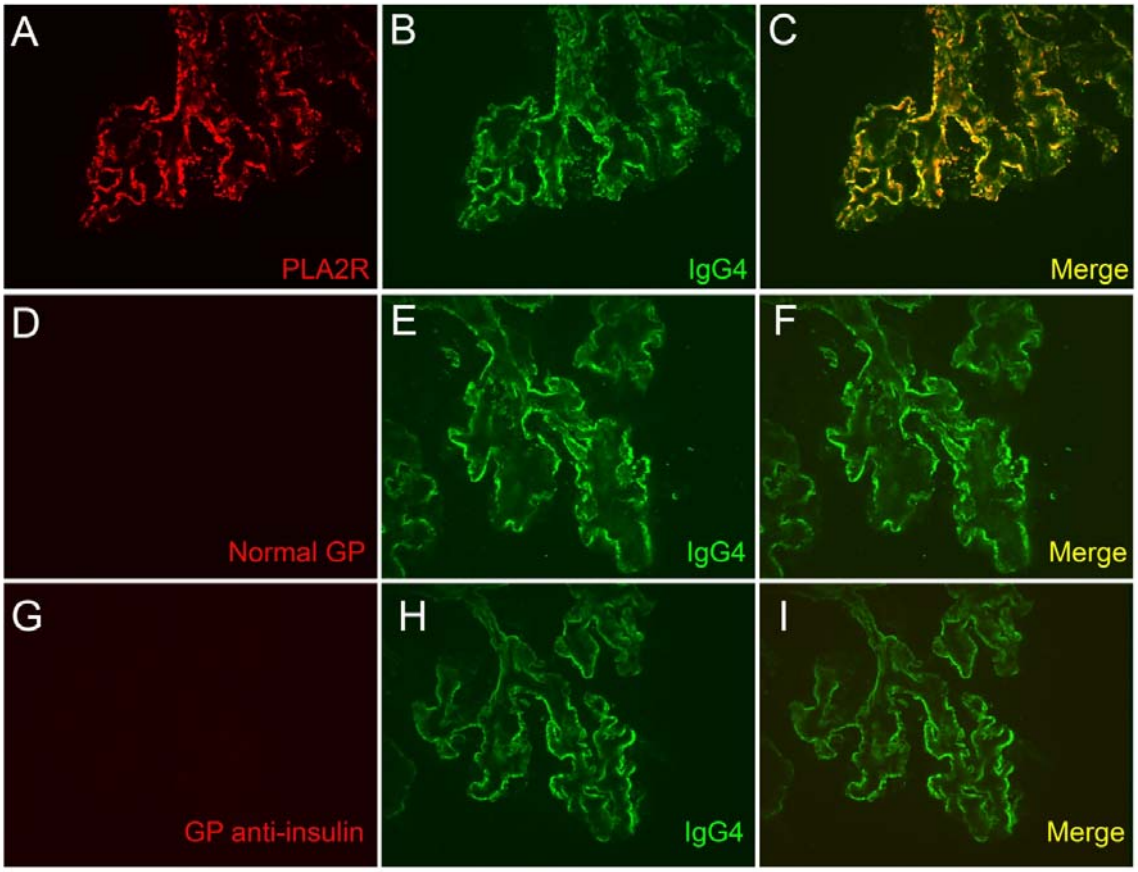
Supplementary Fig. 4: Cultured immortalized podocytes express mRNA and protein for PLA2R. Panel A shows PCR products electrophoresed on an ethidium-bromide stained agarose gel. The expected 605 bp PCR product was found in cultured podocytes in both the growth-permissive (HPOD 33) and differentiation-inducing (HPOD 37) culture conditions. The correct-sized product was also found in normal human glomeruli (Human glom.) and the plasmid containing full-length PLA2R, but not in a reaction to which no template was added. Panel B shows a WB for PLA2R of podocyte lysates from both the 33° C growth-permissive and 37° C differentiation-inducing conditions, showing expression of PLA2R that corresponds in size to rPLA2R as expressed in HEK293T cells.

Supplementary Figure 5



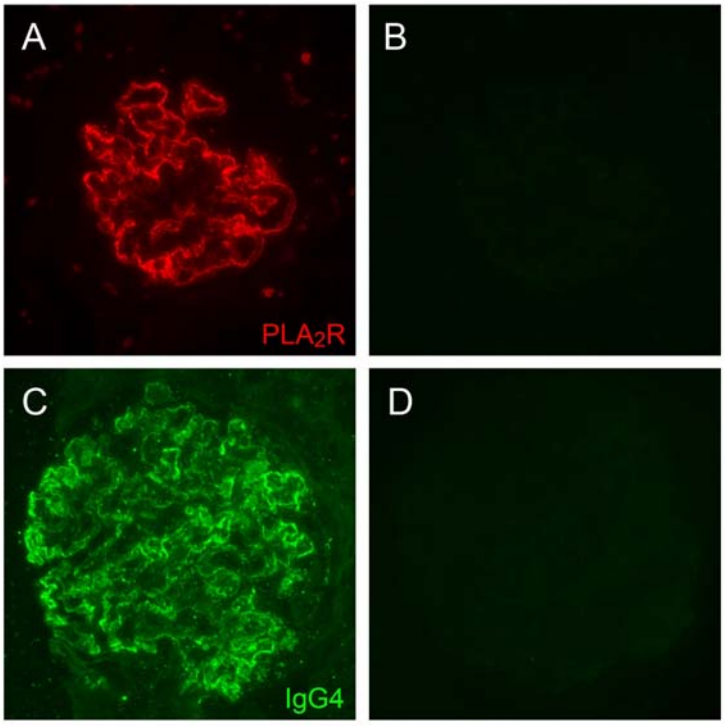
Supplementary Fig. 5: Immunolocalization of PLA2R with respect to smooth muscle actin (SMA; a mesangial cell marker). Frozen sections of normal human kidney were dual stained for PLA2R (1:200, followed by Alexa Fluor 488-conjugated anti-guinea pig IgG at 1:500) as shown in Panel A, or SMA (Cy3-conjugated primary mouse monoclonal antibody, 1:200) in Panel B. As can be seen in the merged image (Panel C), there is no co-localization of the podocytic PLA2R staining and the mesangial SMA staining. Panel D shows a higher power image of another glomerulus stained as above, but with the addition of anti-WT1 at 1:100 followed by Alexa Fluor 350-conjugated anti rabbit IgG at 1:500. The PLA2R-expressing podocytes demonstrate WT1-positive nuclei (arrowheads), whereas the PLA2R-negative, SMA-positive mesangial cells have unlabeled nuclei (arrow). Original magnifications 200x (A-C), 600x (D).

Supplementary Figure 6



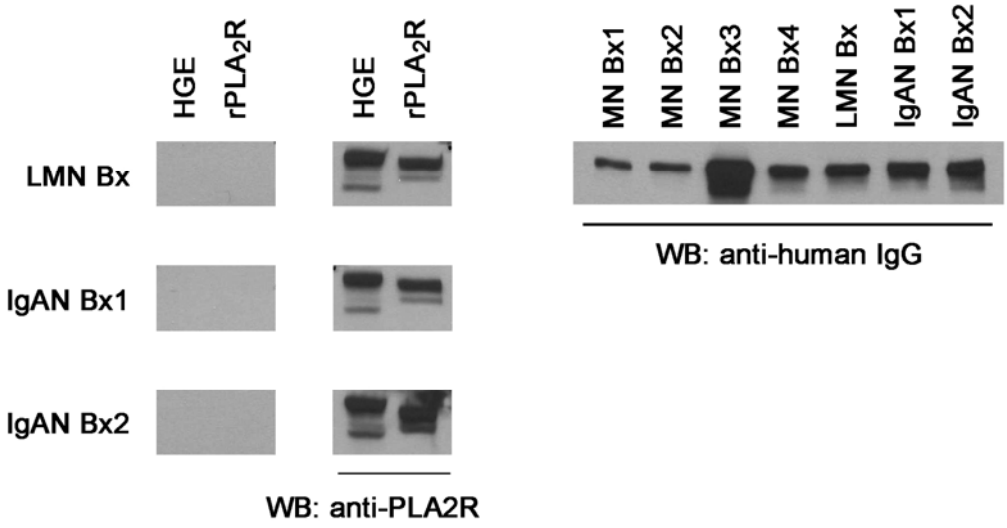
Supplementary Fig. 6: Control confocal microscopic images are presented to supplement Fig. 5A-F of the main text. Panels A-C show confocal images of a glomerular tuft from a MN biopsy specimen that has been stained with anti-PLA2R at 1:150 (A) and anti-IgG4 at 1:500 (B), and the merged image (C) showing co-localization of the two proteins. Panels D-F demonstrate on a serial cryosection of the same MN biopsy specimen that staining with normal GP serum at 1:150 is negative and thus there is no co-localization with IgG4 on the merged image (F). Panels G-I: Use of an irrelevant GP antibody (anti-insulin, at 1:150) is similarly negative on a serial section of the same specimen. Original magnifications 600x.

Supplementary Figure 7



Supplementary Fig. 7: This figure provides additional controls to supplement Fig. 5A-F and Supplementary Fig. 6. Panel A represents an MN biopsy cryosection immunostained for PLA2R and detected with Cy3-conjugated donkey anti-guinea pig IgG. Panel B shows the same glomerulus photographed through the FITC filter to show that there is minimal bleed-through from the Cy3 conjugate that might have otherwise accounted for an apparent co-localization with IgG4. Panel C shows a different glomerulus dual-stained for PLA2R (as above) and for IgG4 with sheep anti-human IgG4 followed by FITC-conjugated anti-sheep IgG, photographed through the FITC filter, showing intense staining of IgG4 and again no bleed-through from the Cy3 conjugate. Panel D shows a glomerulus stained as above, but omitting the anti-human IgG4 primary antibody to show that the FITC-conjugated anti-sheep IgG does not detect the added guinea pig (anti-PLA2R) or donkey (anti-guinea pig IgG) immunoglobulins and does not react with the human IgG present in the subepithelial deposits. Original magnifications 200x.

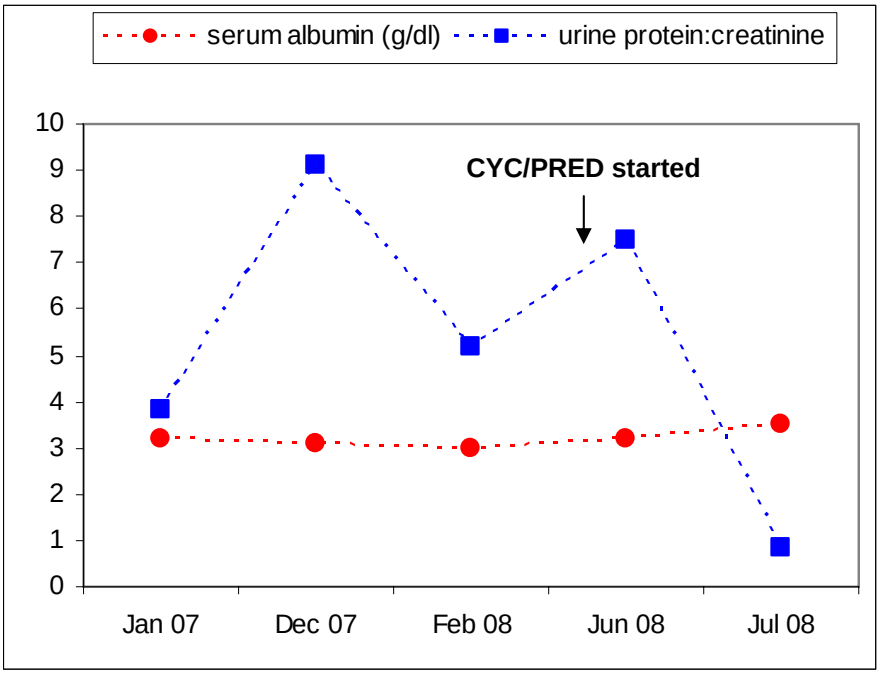
Supplementary Figure 8

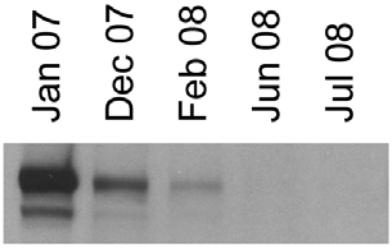


Supplementary Fig. 8: Control figures for IgG elutions from human kidney biopsy cores. In the left panel, the mini-blots that had previously been negative when stained with IgG eluted from LMN or IgAN biopsy cores (Figure 5B of main text) were re-blotted with anti-PLA2R and detected with peroxidase-conjugated anti-guinea pig IgG. In all cases, bands corresponding to native PLA2R and rPLA2R are now detected. The panel at the right shows the relative amounts of IgG eluted from each pair of biopsy cores. Due to the large amount of IgG obtained from the MN Bx3 pair, it was used at a 1:8 dilution to immunoblot the full-length blot of HGE seen in Figure 5J in the main text.

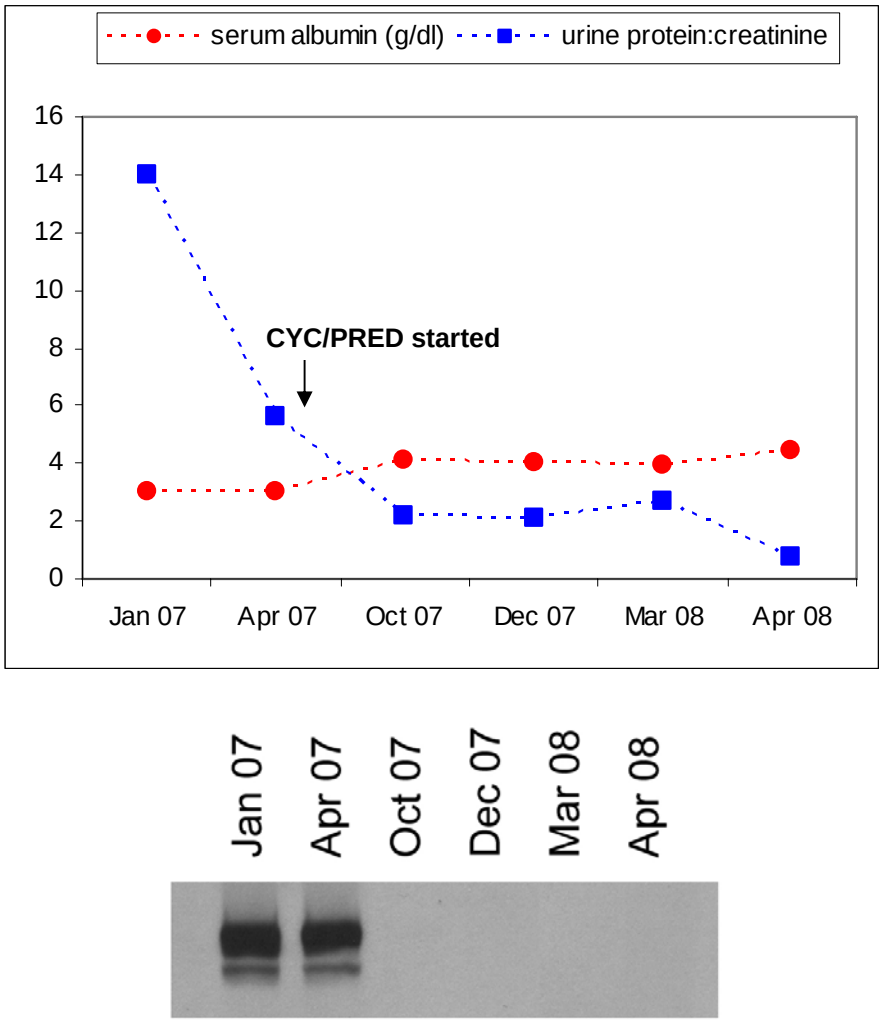
Supplementary Figure 9

A. Patient MN4





B. Patient MN5



Supplementary Fig. 9: Clinical variables (serum albumin and urinary protein) are plotted for two MN patients whose sera were initially positive for anti-PLA2R on WB, and are correlated with immunoreactivity to native PLA2R in human glomerular extracts (HGE). Red circles, serum albumin values in g/dl; blue squares, urinary protein-to-creatinine ratios. Arrows indicate the approximate start date of immunosuppressive therapy with cyclophosphamide (CYC) and prednisone (PRED). For the WBs shown beneath each graph, equal amounts of HGE were immunoblotted with a 1:100 dilution of each serial serum sample, corresponding to the dates on which the clinical parameters were measured. Autoantibodies reactive to PLA2R are detected with peroxidase-conjugated donkey anti-human IgG at 1:40,000.

Clinical Vignettes

A. Patient MN4 is a 54 year-old black female who developed nephrotic-range proteinuria in the fall of 2005 in addition to worsened control of her hypertension and dyslipidemia. Her history is otherwise notable for type 2 diabetes and chronic infection with hepatitis C. She underwent kidney biopsy in December 2006, which showed stage III membranous nephropathy with subepithelial but no mesangial or subendothelial deposits on electron microscopy. She was initially treated conservatively with an angiotensin II receptor blocker (ARB), loop diuretic, and statin, but her proteinuria (estimated by spot protein-to-creatinine ratio) remained in the 4-9 g/d range. Due to a mild worsening of renal function and persistent high-grade proteinuria, she was started on daily oral cyclophosphamide and prednisone in May 2008. By July 2008 her serum albumin had increased to 3.5 g/dl and her urinary protein had fallen to 0.8, suggesting that she was entering clinical remission. As shown by WB analysis, her autoantibodies to PLA2R had already begun to decline prior to immunosuppressive treatment and were undetectable in July 2008.

B. Patient MN5 is a 31 year-old Caucasian male with features suggestive of the nephrotic syndrome a decade prior to his current presentation. He was told he had proteinuria while in college, was diagnosed with hypertension at the age of 24, and noted leg edema 3 years prior to presentation. In June 2006 he was found to have proteinuria at a level of more than 12 g/d, and a kidney biopsy revealed stage II-III membranous nephropathy with 9% of the glomeruli globally or focally sclerosed. He was initially treated with mycophenolate mofetil and oral prednisone, but was switched to cyclosporine and prednisone after failure of the first regimen. Due to persistent nephrotic

syndrome, he was switched to oral cyclophosphamide and prednisone from May 2007 through April 2008, with partial clinical remission occurring in late 2007. Despite this clinical remission, his urinary protein levels persistently stayed in the 2 g/d range. By WB analysis, his autoantibodies to PLA2R had disappeared by October 2007, suggesting his residual proteinuria was not due to immunologically active MN, but due to secondary structural changes in the glomerulus, as were already apparent on his kidney biopsy in June 2006.

C. Patient MN14 (see Fig. 6 of the main article) is a 42 year-old Caucasian male who was diagnosed with membranous nephropathy by kidney biopsy in approximately 1995. He was treated for 6 months with oral cyclophosphamide and prednisone, with resolution of his nephrotic syndrome, allowing him to discontinue all medications. In the 6 to 8 months prior to re-presentation in December 2005 he had noted a 30 pound weight gain and leg edema, and was found to have 5 g/d proteinuria. In addition to treatment with an ARB, loop diuretic, and statin for recurrent membranous nephropathy, he was given a one-year course of oral cyclophosphamide starting in March 2006 combined with 9 months of oral prednisone. He had achieved a complete remission by October 2007.